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14. ABSTRACT Inflammation is now considered a hallmark of Parkinson's disease (PD). As such, it is necessary to understand how this inflammation arises and how it may contribute to the propagation of PD. We and others believe that synuclein may be the cause of this inflammatory response due to its release into the extracellular space. Here, we used immortalized N9 cells and N(/CD36 deficient microglial cells in our studies on synuclein-induced inflammation. Furthermore, we clarified some of our results in paraffin-embedded substantia nigra pars compacta (SNpc). Using the aforementioned cells and several different cell culture experiments with wild-type (WT) and mutated synuclein, we found that both types of synuclein increase microglial adhesion and decrease microglial migration. Several antibodies inteferred with the synuclein effects by blocking the synuclein-induced decrease in migratory behavior and increase in adhesive behavior of the microglia. Using WT fluorescent synuclein-coated nanobeads, we saw that synuclein not only stuck to the surface of the microglia, but was also internalized by the microglia. We believe that this occurred through CD36 and CD11b. Since we saw that our results were not 100%, we believe that other scavenger receptors and integrins are involved. Immumostaining of the SNpc showed a robust staining for CD36. We are now working on a two color immunostaining for CD36 localization in which cells. From these studies, we conclude that CD36 and CD11b are major players in the inflammatory response induced by the synucleins^.

15. SUBJECT TERMS
Inflammation, synuclein, scavenger receptors, beta-integrins, CD11b, CD36, Parkinson's Disease

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Interaction of Synuclein and Inflammation in Dopaminergic Neurodegeneration

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Introduction

Parkinson Disease (PD) is the second most common progressive adult onset neurodegenerative disorder behind Alzheimer Disease. It is characterized clinically by resting tremor, slowness of movement, muscle rigidity and postural instability, all attributed to, mainly though not exclusively, the loss of the dopaminergic neurons in the substantia nigra pars compacta (SNpc) and their dopaminergic terminals in the corpus striatum of the nigrostriatal pathway in the brain (Dauer and Przedborski, 2003). To a lesser extent, other transmitter systems such as the noradrenergic and the serotonergic systems are also affected (Dauer and Przedborski, 2003). Also found within the remaining dopaminergic neurons in the SNpc in PD are proteinaceous interneuronal inclusions called Lewy bodies. These bodies, eosinophilic cytoplasmic aggregates, are composed of a variety of proteins such as α-synuclein, ubiquitin, parkin, and neurofilaments and seem to be necessary for the definitive diagnosis of PD (Dauer and Przedborski, 2003). However, the reason for their presence remains elusive. Recently, studies of the SNpc of those individuals, who had self-administered MPTP, revealed activated microglia within the SNpc up to sixteen years after administration of MPTP (Langston et al, 1999; Barcai et al, 2004). Thus, neuroinflammation is now considered a hallmark of PD and, it is thought that the noted inflammation results from dying dopaminergic neurons releasing their contents into the extracellular space of the cell (Surmeier and Sulzer, 2013). One of the substances that is released is alpha-synuclein thus, we and others think that there is a possibility that the root cause of the noted inflammation seen in the SNpc of PD brains may indeed be the neuronal release of alpha-syn. Since this is the thinking, the question now is: how is neuroinflammation tied to the presence of α-synuclein in the SNpc?

The synuclein protein is well-conserved across mammalian species (Maroteaux et al, 1988), is conspicuously expressed throughout the brain and has been identified in close proximity to synaptic vesicles in presynaptic terminals (Maroteaux et al, 1988; Iwai et al, 1995). To date, three missense mutations in α-synuclein (A53T, A30P and E46K) have been noted in PD and these mutations as well as the over-expression of wild-type α-synuclein have been linked to the familial form of PD (Polymerpoulos et al, 1997; Kruger et al, 1998; Masliah et al, 2000; Zarranz et al, 2004;) and, more importantly, to an early-onset form of the disease. Both the A53T and the A30P mutants increase the rate of α-synuclein fibrillation (Bennett, 2005) and the formation of β-sheets (Narhi et al, 1999) while, the overexpression of WT α-synuclein in a number of cell types can result in aggregate formation (Lee and Lee, 2002). Post-translational modification of WT α-synuclein, such as nitration (Przedborski et al, 2001) and oxidation (Hashimoto et al, 1999), can lead to structural changes in the cells, and if these are not properly cleared, they may give rise to a neurotoxic event within the cells.

The nature of the extracellular SN environment has profound implications in PD. Studies in humans show that the release of α-syn into leads to the presence as syn oligomers in plasma (El-Agnaf, Salem et al. 2006) and CSF (Mollenhauer, El-Agnaf et al. 2010; Mollenhauer, Locascio et al. 2011). Indeed, other studies show that Lewy bodies appear in fetal transplanted tissue in PD individuals between 3-14 years after receiving fetal tissue transplants. Thus, the appearance of Lewy bodies in

fetal tissue is consistent with extracellular environmental changes inducing pathology in the SN in PD (Zecca et al, 2008). Moreover, injection of neuromelanin into the SN of rats causes α-syn to cluster within individual SN neurons (Zecca, et al, 2011). The exact mechanisms by which the presence of native or modified α-syn or the overexpression of α-syn in mouse models of PD leads to neurodegeneration is not completely understood. Similarly, the propensity of this protein to misfold into toxic oligomers that may lead to PD (Kalia, Kalia et al. 2012; Pieri, Madiona et al. 2012) remains to be elucidated.

Since neuroinflammation is now a recognized hallmark of the PD morphological picture, a very important question is, how are microglial cells activated in PD? That the observed activation of glial cells in neuroinflammation in PD may be the cause of the progressive nature of the disease is the present theory but, this remains to be clarified. However, we do know that, on autopsy, activated glial cells are present in the SNpc of PD brains and there seems to be evidence of an on-going active degenerative process in this brain area (Langston et al, 1999). We also know that pro-inflammatory components, such as interleukin 1β (IL-1β) and prostaglandin PGE_2, are increased in the SNpc and CSF from PD patients (Mogi et al, 1994, 1996, 2000). In PD models, activated glia are present in the SNpc, along with markers of inflammation, such as elevated levels of NADPH oxidase (Wu et al, 2002), inducible nitric oxide synthase (iNOS) (Liberatore et al, 1999) and PGE_2 (Teismann et al, 2003). Our interesting finding of the presence of post-translational modified α-synuclein (Przedborski et al, 2001) within the DA neurons of the SNpc points to the possibility that a connection exists between this finding, oxidative stress and neuroinflammation as nitrated synuclein is most likely a permanent fingerprint whose insolubility may initiate the noted neuroinflammation. Previous work with an injection of neuromelanin into the SNpc of rats resulted in an inflammatory response plus increased synuclein and ubiquitin accumulation within the DA neurons of the SNpc (Jackson-Lewis et al, 2008). This demonstrates that if enough of an accumulation of synuclein occurs within the DA neuron, this may cause the cell to rupture, spewing its contents including the synucleins in their various forms and modifications, into the extracellular space. And, it has been demonstrated, at least in the test tube, that synucleins can activate microglial cells via some signaling pathways that are, at present, not totally known or understood..

During the early part of our investigations, we injected alpha-synuclein (5 micrograms/microliter for a total of 20 micrograms) into the SNpc of rats and noted that this particular dosing schedule, during this time-course experiment, destroyed SN tissue. Following a reduction in the syn concentration to 8 micrograms in 4 microliters (about a year or so), we noted, in our in vivo studies, that the inflammatory response peaked at two days after the synuclein injection. For this time-course, we performed immunostainings of the rat ventral midbrain for various inflammatory markers from 0 to 21 days after the synuclein injection and all of the inflammatory markers (Iba-1, COX-2, GFAP, iNOS, NFkB, etc) were up-regulated during the time-course study, the strongest response being between 2 days and four days after the syn injection. In our in vitro studies, we found that that the beta-integrin, CD11b, and the scavenger receptor CD36 mediate the adhesion and migration of microglia to matrix-bound and modified synuclein.

In chasing this synuclein-induced inflammatory response, for the past year, we have focused a significant part of our efforts on the finer points of the inflammatory response by examining, further, the roles of beta-integrins and scavenger receptors in the initiation of the synuclein-induced inflammatory response. For this, we used cell culture techniques employing immortalized N9 and N9/CD36-deficient murine microglial cells because these experiments require millions of cells per assay and primary murine microglial cell numbers were not increasing fast enough. Thus, we have used these immortalized N9 cells and N9/CD36 knockout cells to examine the roles of CD11b and CD36 in microglial cell adhesion, migration and chemotaxis across synuclein-containing matrices.

Body of Research

The overall goal of this part of the synuclein project is to show how the synucleins, be they wild-type, oxidized, mutated, or nitrated, can initiate an inflammatory response within the SNpc of rats. In this respect, this necessitates the examination of individual events that lead to the inflammatory response caused by the synucleins. To this end, we are assessing the effects of extracellularly applied α-synuclein on glial proliferation and motility, as we believe that post-translationally modified and mutated alpha-synuclein species may provoke the clustering of glial cells around the site of the intracerebral synuclein injection. The degree of the clustering may be dependent on the type of synuclein applied. In magnitude, this inflammatory response parallels the known pathogenicity of a range of different synuclein species, and is likely due to a combination of key features of the glial response, namely proliferation and chemotaxis.

Our *in vivo* studies have demonstrated that injection of alpha-synuclein into the SNpc of rats does indeed cause an inflammatory response whose morphological picture mimics PD. Events that are characteristic of an inflammatory response, including the production of cytokines and chemokines are also demonstrated. In parallel, in the *in vitro* aspect of this study, glial cultures were exposed to the same set of α-synuclein species and their effects on glial chemotaxis and proliferation compared. Because there is a literature on the biochemical interaction between DA and synuclein (Lee et al, 2011; Ulusoy et al, 2012), we assessed whether the synucleins can stimulate the production of reactive oxygen species (ROS) and/or nitric oxide (NO) using both in vivo and in vitro techniques and glial markers, immunostaining, and confocal microscopy. To continue our story on alpha-synuclein and inflammation, we asked what is the mechanism by which cell surface receptors interact with synucleins to provoke the inflammation that is so characteristic of PD and other neurodegenerative disorders? Getting a handle on this may reveal therapeutic targets that can be used to possibly dampen or eliminate glial activation and, thus, PD disease progression.

N9 cells, like other immortalized microglia cells, chemotax poorly to known chemoattractants such as GMCSF. We, therefore, selected a subpopulation of N9 cells that migrated across laminin-coated membranes in cell culture inserts for these studies. A subpopulation of N9 cells is regenerated every two months by maintaining and growing those few N9 cells (less than 1%) that migrated in response

to ng concentrations of GMCSF. In response to GMCSF, a two-three-fold increase in these migratory N9 cells across matrix-bound laminin is observed). For these experiments, 3 ug of murine laminin were deposited onto PD cell culture 8 micron chemotaxis chambers. N9 cells (10^5) were placed in the upper compartment and the chemoattractant placed in the lower compartments. The cells were allowed to migrate for 24 hours, at which time, 100 ul of trypsin/EDTA is added to the upper chamber. The typsin/EDTA detaches cells that have migrated to the underside of the membrane into the lower compartment without causing more cells to migrate across the protein coated filters. The inserts are removed and the detached migratory cells are allowed to adhere to the lower compartment of the 96 well plates. After several hours, the number of cells is determined using a CyQuant cell adhesion assay. Here, we show that N9 migration is significantly reduced by greater than 50% when native synuclein is allowed to adhere to laminin-coated cell inserts. Equally important, we see a small but significant further reduction in N9 cell chemotaxis across native synuclein in the presence of antibodies directed against CD11b or anti- CD36. Matrix-bound nitrated synuclein and mutated synuclein are more efficient at blocking N9 chemotaxis in response to GMSCF as compared to native synuclein. We show that the presence of matrix-bound nitrated synuclein reduces chemotaxis by greater than 75% and again, both anti-CD11b and anti-CD36 antibodies further reduce chemotaxis. A similar pattern is observed when we examined chemotaxis across A53T mutant synuclein.

We obtained similar results under conditions where the cell inserts were only coated with native or modified synuclein. Currently, we are maintaining and growing up those few N9 cells that have migrated across synuclein-coated surfaces and we will examine their chemotaxic properties in the absence or presence of anti-CD11b or anti- CD 36 antibodies. These studies extend our results on N9 adhesion by showing the functional effects of blocking CD11b integrins and scavenger receptors on cell chemotaxis. We are now beginning to examine the effects of these antibodies on the uptake of soluble synuclein peptides.

We are also looking into what mammalian cells take up the human synuclein: DA neurons, microglia or both. To assess this, we are now in the process of immunostaining the rat SNpc tissues from the time course study for human synuclein. Following this, we will use microglial cultures, both primary and immortalized, to examine whether microglia take up the human synuclein and, if so, how does this process works?

CD36 and CD11b serve as primary receptors for mediating the adhesion and chemotaxis of microglia with matrix-bound synuclein. We continue to examine the roles of the integrin CD11b and the scavenger receptor CD36 as primary receptors that mediate the interactions of microglia with synuclein. N9 microglia that are deficient in CD36 were generated by our collaborator, Dr. Joseph El Khoury. These CD36-deficient N9 cells are less responsive to the inhibitory effects of anti-CD36 antibodies. Wild type N9 cells that chemotax across synuclein matrices and adhere to the matrices are blocked by these antibodies. In contrast, the extent of inhibition of N9 CD36- deficient cells by these antibodies is reduced. Yet, adhesion to synuclein remains strong because these cells still express other scavenger receptors such as SR1 and RAGE, which we intend to investigate.

We also have compelling data that show that N9 cells exhibit increased adhesion to two forms of mutated synuclein, A53T and A30P. Adhesion to the mutated forms of synuclein is also mediated by both CD36 and CD11b. As expected, CD36-deficient N9 cells are less responsive to the blocking effects of anti-CD36 antibodies. The CD36 inhibitor, ursolic acid, at concentrations of 10^{-5} and $10^{-6,}$ reduced N9 chemotaxis across native synuclein by almost 50%. Equally interesting is the fact that ursolic acid had a small effect on CD36-deficient N9 cell chemotaxis across synuclein which suggests that ursolic acid may also inhibit other scavenger receptors such as SR1 and RAGE. Ursolic acid was examined because Dr. El Khoury (1996) showed that ursolic acid dramatically blocked the CD36-mediated adhesion of microglia cells to beta amyloid matrices and reduced neurodegeneration in mouse models that mimic Alzheimer's disease. We have clarified these results and will therefore examine whether ursolic acid can reduce neurodegeneration in a mouse model (where synuclein is overexpressed).

We have begun the another phase of our project which is to examine the roles of CD36 and CD11b in the uptake of soluble synuclein by N9 cells and by CD36-deficient N9 cells. To this end, we have prepared fluorescent synuclein (simply by incubating synuclein with Alexaflour 488). We are at the time-course stage and once we have a handle on fluorescent synuclein uptake and this relationship to time, we will measure cell-associated fluorescent synuclein within the microglia following the incubation of fluorescent synuclein with N9 cells and CD36- deficient N9 cells. We will examine whether anti-CD36 or antiCD11b antibodies block cell-associated uptake of soluble synuclein. These are important experiments in that we need to explore whether soluble synuclein uptake is mediated by the same receptors that mediate adhesion to matrix- bound synucleins. In our first set of studies, just to see if there was WT-synuclein within the microglia, using immunostaining, we found no WT-synuclein within either N9 or CD36-deficient N9 cells. This study was performed several times to clarify our results. We are now incubating the microglia with WT-fluorescent synuclein to gauge the time-dependency of the uptake of synuclein.

In addition, we are using fluorescent synuclein to examine the capacity of N9 cells to clear matrix-bound synuclein. In these experiments, fluorescent synuclein is bound to the surface of 96 well plates and then N9 cells are added for various times (1h to 7 days). The cells are then removed by the addition of EDTA and the remaining fluorescent synuclein is measured in a cell plate reader. Furthermore, the fluorescence present on the 96 wells and in the detached cells will be assessed using fluorescent microscopy. Preliminary experiments show increased clearance of matrix-bound fluorescent-synuclein by N9 cells after 48 hours. We are working out this assay and once this assay has been worked out, we will examine the roles of CD36 and CD11b as described above.

We are also attempting to engineer N9 cells that overexpress CD36 to see the effects on a) adhesion, b) chemotaxis, c) synuclein clearance and d) soluble synuclein uptake. Once we have these cells and obtain these results, we will look into genetically designing both a CD36-deficient and a CD36 overexpressing mouse, cross these with synuclein overexpressers and then examine these mice behaviorallymeasure neurodegeneration in the crosses. These types of experiments might provide

insights in designing interventions that reduce the neurodegeneration seen in PD.

In our efforts to sort out what is involved in the inflammatory response to synuclein *in vivo,* we perfused mice engineered to overexpress wild-type alpha-synuclein and their age-matched normal control mice with saline followed by 4% Paraformaldehyde (PF) in 0.1M phosphate buffer (PB), pH 7.2-7.4. Brains were quickly removed, post-fixed overnight in the same fixative, cryoprotected in 30% sucrose in 0.1 M PB, frozen in dry ice-cooled isopentane and stored at -80ºC until used. For immunostaining, brains were sectioned in series at 30 μm and the entire midbrain and striatum were collected free-floating in 0.1M PB containing 0.01% sodium axide (4, 5). Mice were ~1.5 years of age and presented with a slight ataxic gait and a less than normal tail suspension test.

Due to reconstruction and modernization of our present animal housing area by the university, mouse housing for our mice was moved to a new building. The new animal housing area is a large bio-safety level 3 (BSL3) suite because of our use of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) as a mouse model of PD. This suite also contains our surgery suite for mice. Rat housing for our rats has also been moved and we are now in the process of getting a new surgery/behavior room that also houses our rats.

Key Accomplishments

***In Vitro* Studies**

We have developed a method to label synuclein with Alexa 488 to gauge whether microglia take up synuclein. This method is quite simple and straightforward in that we just incubate synuclein with Alexa 488. However, prior to the incubation of microglia with Alexa 488, we incubated microglia with WT synuclein and then immunostained the microglia for the presence of synuclein. After ascertaining that microglia do take up synuclein via immunostaining, we then used coated 96 well plates with 3 ug of laminin, 1ug of Fluorescent-labeled (Fl) synuclein and 2 ug of unlabeled synuclein per well. Each well was washed with PBS to remove unbound protein and 50,000 N9 microglial cells were added to each well in the presence of GMCSF. After 2 hrs, the wells were washed again with PBS and 100 ul of 10 mM EDTA was added to each well to remove all of the adhered cells. The residual fluorescence in each well was measured using a cell plate reader. The amount of synuclein cleared from each well was then calculated by subtracting the amount of residual fluorescence at 2 hr from a zero hour reading. In duplicate experiments using 4 wells per condition, we observed that 30% ± 1% of the fluorescence was removed or cleared by the N9 microglial cells. In contrast, when N9/CD36 knockout primary murine microglial cells were used, only 20% ± 0.5% of the fluorescence was cleared by the cells. From our data, it appears that N9 cells have the capacity to take up and clear synuclein from the matrix. It also seems that CD36 appears to be an important cell surface receptor involved in this process.

We are examining the effects of various antibodies (anti-CD36, anti-CD11b, anti-CD18, anti-SR1, and anti-RAGE) to substantiate the roles of CD36 and CD11b integrins on synclein clearance. In addition,

we added ursolic acid to these studies and examined its effects as it has been shown that this compound blocks CD36 in microglial cells (El-Khoury et al, 1996) and can block the clearance of beta-amyloid from a protein matrix We envision that both ursolic acid and several of the pattern recognition receptors will also block synuclein clearance. These data reveal important information regarding the mechanism by which normal microglial cells clear extracellular synuclein from their CNS microenvironment.

In conjunction with these experiments, we are using the fluorescence–labeled synuclein to examine synuclein uptake by microglia. In our proposed experiments, we allow N9 cells to adhere to laminin-coated surfaces in 96 well plates and then add soluble fluorescent-labeled synuclein for 0, 2, 6 and 24 hr. The wells are then washed and the residual cell-associated synuclein is measured using a cell plate reader. We will confirm the cellular uptake of synuclein by N9 cells using fluorescent and confocal microscopy. This method will allow us to examine synuclein uptake and distribution by CD36 knockout primary murine microglia as well as the action of the above-mentioned antibodies on the synuclein uptake by microglia. Preliminary data shows that microglia do indeed take up synuclein. We are also examining the effects of ursolic acid on synuclein uptake.

In Vivo Studies

In our efforts to sort out what is involved in the inflammatory response to synuclein *in vivo,* we perfused mice overexpressing wild-type alpha-synuclein and their matching wild-type control mice with saline followed by 4% Paraformaldehyde (PF) in 0.1M phosphate buffer (PB), pH 7.2-7.4. Brains were quickly removed, post-fixed overnight in the same fixative, cryoprotected in 30% sucrose in 0.1 M PB, frozen in dry ice-cooled isopentane and stored at -80ºC until used. For immunostaining, brains were sectioned in series at 30 μm and the entire midbrain and striatum were collected free-floating in 0.1M PB containing 0.01% sodium axide. Mice were ~1.5 years of age and presented with a slight ataxic gait and an abnormal tail suspension test.

To examine the effects of synuclein overexpression in a transgenic mouse, we immunostained (IHC) the ventral midbrain containing the substantia nigra (SN) and the ventral tegmental (VTA) areas, using our standard IHC protocol, for the following: Tyrosine Hydroxylase (TH), ubiqiutin (Ubiq) and alpha-synuclein (α-syn). For the markers of inflammation, we immunostained the same tissue areas for activated microglia (CD11b), beta-integrins (CD36) and astrocytes (glial fibrillary acidic protein, GFAP). In scanning the slides from the alpha-synuclein overexpressing brains, we found: 1) activated microglia in the SN, which were present but not full-blown; 2) activated astrocytes in the SN, which were quite prominent; 3) microglia that were CD36+positive in the SN compared to their nontransgenic littermates. The sections from the alpha-synuclein overexpressors were positive for synuclein and for ubiquitin compared to the non-transgenic controls. Stereology will be used to count the dopaminergic neurons in the SN to gauge the differences in neuron numbers between the two groups of mice. As we are trying to elucidate what causes the inflammation in PD, we try to replicate what we see/find in our animal models.

We have also immunostained paraffin-fixed human substantia nigra for the localization of the beta-integrin CD 36. The immunostaining showed the presence of CD36 but, we could not say for sure

whether neurons or microglia contained the CD36. Thus, to demonstrate clearly the localization of CD36 and to increase our numbers of human SN tissues to an N of three per group, we are now working out a double staining procedure to clarify exactly where CD36 is located. Below is the procedure we have used for this since the SN tissue we are using is paraffin-fixed.

Double staining procedure for CD-36 with Vector SG and DAB and TH (Calbiochem) with DAB for 8 microns, slide-mounted SN sections.

Prior to the immunostaining procedure, it is necessary to deparaffinate tissues. Thus, the following is used:

1. Place slides in a 60ºC oven for at least 60 mins, then process tissues as follows:
2. Xylene: 3 X 10'
3. Alcohol 100%: 2 X 3'
4. Alcohol 95%: 2 X 3'
5. Alcohol 75%: 1 X 3'
6. Alcohol 50%: 1 X 3'
7. Rinse in DW (distilled water): 1 X 5'
8. PBS (phosphate buffered saline)/TBS (tris buffered saline: 2 X 5'
9. H_2O_2 (Hydrogen Peroxide) in PBS/TBS 30' (3.3 mL of 30% H_2O_2 in 96.7 mL of PBS)
10. Wash in PBS/TBS: 2 X 5'
11. PBS/TBS with 0.1% Triton x100: 2 X 5
12. Apply 3% Normal Goat Serum (blocking serum) in PBS/TBS for 30'
13. Primary Polyclonal (CD36) Ab 1:100-200 and Primary Polyclonal Tyrosine Hydroxylase Ab 1:2000 in PBS/TBS with 3% NGS Over Night on +4^{o}C

Day 2

10 PBS/TBS with 0.1% Triton x100: 2 X 5'

11 Biotinylated AB (Goat Anti Mouse) Place the secondary antibody: 1:200 Biotinylated Goat Anti Mouse + 3% NHSG (or 2% BSA 5%) in PBS/TBS for 1 hour at RT.

12 PBS/TBS <u>without Triton x100:</u> 2 X 5'

13 ABC for 1 hour (1:600 µl reagent A + 1:600 µl reagent B) in 5 mL PBS/TBS diluted at least 30' earlier and left on room temperature.

14 PBS/TBS 2 X 5'

15 DAB: Glucose generation system: 17 minutes

18 PBS/TBS: 2 x 5'

After the immunostaining, tissues were dehydrated, cover-slipped, air-dried and viewed under a light microscope. We are now working on a two-color immunostaining technique to demonstrate exactly where CD36 is located, in the DA neurons or in microglia, or in both.

Reportable Outcomes

•Interaction of Microglial Cells with Matrix bound α-syn: **Adhesion, ROS production, and Chemotaxis**

For most of the experiments in this paper, we choose to use either immortalized N9 or BV2 murine microglial cells for three reasons. First, obtaining sufficient primary murine microglial cells would require the sacrifice of hundreds of neonatal mice. Second, PD is an adult onset disease and the roles of adult glial cells differ from neonatal in diseases like Alzheimer's disease (Husemann et al., 2001). Moreover, obtaining sufficient numbers of adult microglial cells is technically even more difficult than obtaining neonatal cells, as the yields are quite low per mouse sacrificed. Third, we can more easily genetically manipulate or select specific populations from microglial cell lines (especially from N9 cells) than from primary cells.

Lam was used as our control matrix for several reasons. Lam is the primary extracellular matrix in the CNS and unlike other matrix proteins, such as fibronectin and vitronectin, it does not activate microglia cells (Milner and Campbell, 2003). In addition, we compared microglial cell interaction to surfaces coated with lam alone versus surfaces coated with both lam and α-syn. These control experiments showed that N9 cells adhere to surfaces coated with lam and lam/α-syn in proportion to the amount of protein used to coat the wells. Maximum cell adhesion occurred at 3 µg per well for lam and 2 µg per well coated with α-syn overlaid onto the lam. Cell adhesion of both types of transformed microglial cells (BV2 and N9) was greater on α-syn-coated wells as compared to uncoated or lam-coated wells (Table I). We also examined several other properties (such as chemotaxis, TNF production, and ROS production) resulting from the interaction of microglial cells with matrix bound α-syn as compared to matrix bound lam).

Chemotaxis experiments using BV2 or N9 cells were initially difficult to perform because these cell lines migrate poorly across lam-coated surfaces (data not shown), even in response to chemoattractants such as GM-CSF or MCP-1. We therefore, selected a stable subpopulation of N9 cells that are significantly more migratory in response to GM-CSF. These cell subpopulations were selected by growing up those few cells (less than 1%) that migrated in response to GM-CSF across lam-coated inserts after a 48 hr chemotaxis period. The increased capacity of this selected N9 cell population to chemotax across lam-coated inserts continued to be observed even after 20 passages (data not shown). After 20 passages, new chemotactic N9 cell populations were generated as described above.

Approximately 10% of the N9 cells chemotaxed across lam-coated cell culture inserts in response to GM-CSF added to the bottom compartment after a 24 h incubation period. Control experiments showed that less than 3% migrated in the absence of GM-CSF. When α-syn was layered onto the lam-coated cell culture inserts, we observed a dramatic reduction in chemotaxis almost to those values observed in the absence of GM.

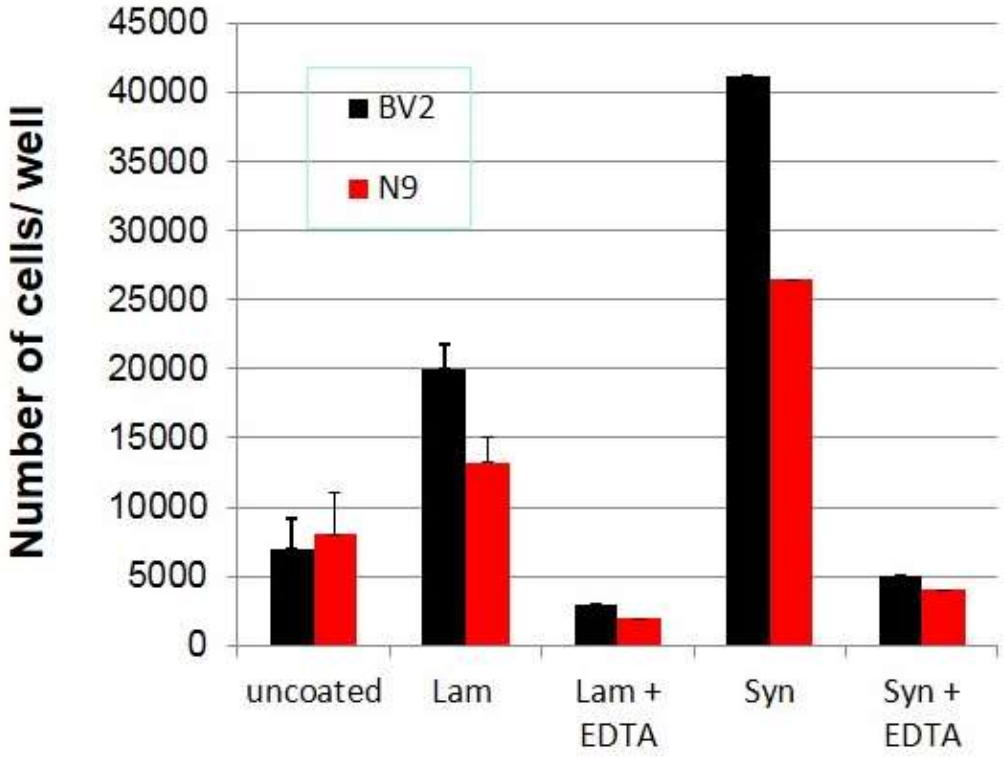
TNF production as measured in the extracellular media was assessed using ELISA technology as described (Braghin et al, 2009). Low levels of TNF (100 pg/10^5 cells) were produced when N9 cells were incubated with matrix bound laminin. However, the amount of TNF produced by N9 cells increased almost 5 fold (500 pg/10^5 cells) when cells were incubated in wells coated with both lam and α-syn . ROS production was also assessed and found to be increased 2 fold in response to LPS when N9 cells adhered to either laminin or α-syn matrix bound surfaces (Table I).There was no clear or significant difference in ROS production of N9 cells adhering to lam vs lam/α-syn coated surfaces.

Table I- The effects of N9 microglial cell interaction with matrix bound lam and α-syn. Adhesion, chemotaxis, TNF production, and ROS production were assessed after N9 microglial cells were allowed to interact with either lam-coated surfaces or surfaces coated with both lam and α-syn as described under Materials and Methods. Adherence is measure as the percent of added cells that adhered after a 2 hr incubation. Chemotaxis reflects the percent of cells added to the upper chamber of the cell insert that migrated into the lower compartment. TNF production is expressed as pg/10^5 cells and ROS production is expressed as arbitrary units.

Microglia Cell Interactions	Laminin	Synuclein
Adhesion	30-40%	70-80%
Chemotaxis	10%	3-4%
Cytokine production (TNF)	100 pg/10^5 cells	500 pg/10^5 cells
ROS Production [LPS/no LPS (90 min)]	2 fold increase	2-3 fold increase

•**Adherence:** In order to identify potential cell surface receptors responsible for microglial cell adhesion we first assessed the effects of the Ca^{++} chelator, EDTA, in our adhesion assay. N9 Cell adhesion was shown to be dependent on the concentration of lam-coated surfaces with cell adhesion maximizing at a lam concentration of 3 µg/well (data not shown). Similarly, cell adhesion to matrix-bound α-syn peaked at protein concentrations between 1-2 µg of α-syn added per well. Within the two hour adhesion assay about 30-40% of the cells adhered to lam-coated surfaces. More than twice that number of N9 cells adhered to surfaces coated with both α-syn and lam (Table I). Incubating the cells in the presence of 5 uM EDTA dramatically reduced the capacity of N9 cells to adhere to either matrix bound lam or α-syn-coated surfaces. Similar results were obtained using BV2 murine microglial cells (Figure I).

Figure 1. Adhesion of BV2 and N9 cells to α-syn-coated surfaces. Wells were either uncoated or coated with the indicated protein and 10^5 cells were added to each well as described in Materials and methods. As indicated, some wells contained 5 mM EDTA. Cell adherence was measured after 2 hr using a CyQuant assay as described in Materials and Methods. Fluorescence was read using a Cytoflur II plate reader. Each data point is the mean of quadruplicates from a representative experiment repeated three times. Error bars, SEM.



•**Effect of anti-integrin antibodies on microglial cell adhesion**. Cell surface integrin adhesion is dependent upon divalent cations (Zhang and Chen, 2010). The observations that EDTA dramatically blocks N9 and BV2 cell adhesion to both lam-and lam/ α-syn-coated wells suggest that adhesion may be mediated by cell surface integrins. We therefore examined the effects of various anti-integrin antibodies against either the beta or alpha subunits of integrins on microglial cell adhesion to lam- or lam/ α-syn–coated surfaces (Figures 2a, 2b). Anti-beta$_1$ integrin antibody slightly inhibited (15%) BV2 cell adhesion to lam- or lam/α-syn–coated surfaces. This is consistent with others (Milner and Campbell, 2002) who showed that anti beta$_1$ antibodies blocked BV2 cell adhesion to lam-coated surfaces. In contrast, anti-beta$_2$ integrin antibodies blocked BV2 cell adhesion to lam/α-syn – coated surfaces by about 30% (Figure 2a).

Figure 2a.

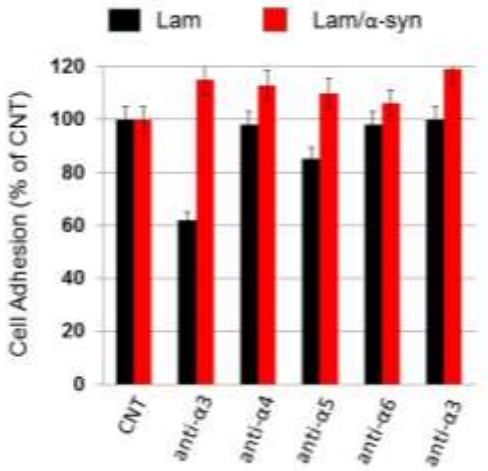
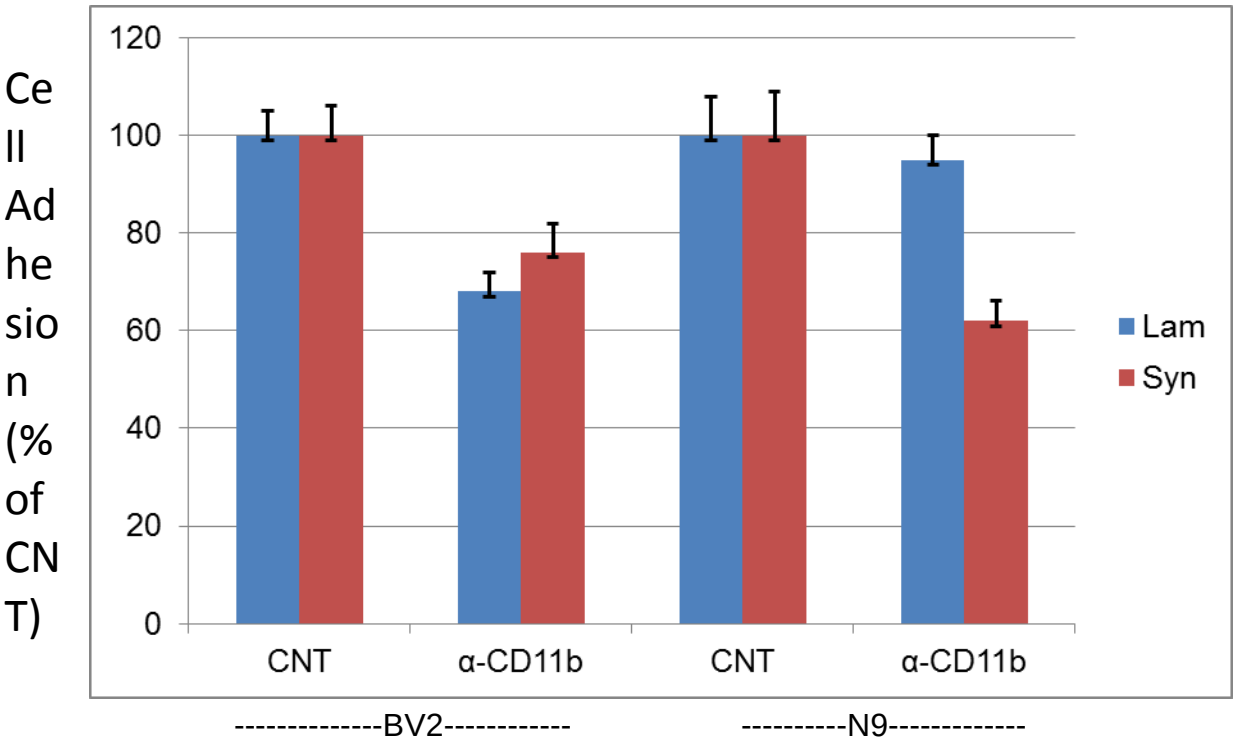


Figure 2b: Effects of anti-integrin antibodies on the adhesion of microglial cells to matrix- bound lam and α-syn. BV2 cells (50,000 cells per well) were added to 96 well plates coated with lam and α-syn as described under Materials and Methods. To some of the wells 10 µg per ml of the indicated antibody was then added. After a 2 hr incubation, each well was washed gently with 200 µl of PBS and the number of remaining adherent cells were assessed as described under Materials and Methods.

Because there are different subclasses of alpha subunits associated with beta integrins, we examined the effects of anti-alpha subunit integrin antibodies on microglia cell adhesion to lam- or lam/α-syn–coated surfaces (Figure. 2b). Anti α3 and anti α5 (associated with β1 integrins) showed small but significant effects on cell adhesion to lam-coated surfaces. No other anti α-antibodies tested affected BV2 cell adhesion (Figure 2b). However, we observed that anti CD11b (or αM subunit that is associated with β2 integrins) blocked cell adhesion of BV2 microglia to both lam- and lam/α-syn–coated surfaces (Figure 2c). To confirm the role of CD11b antibodies on BV2 microglia cell adhesion to lam- and lam/α-syn –coated surfaces, we show that this antibody also blocks N9 cell adhesion to these matrix bound proteins (Figure. 2c).

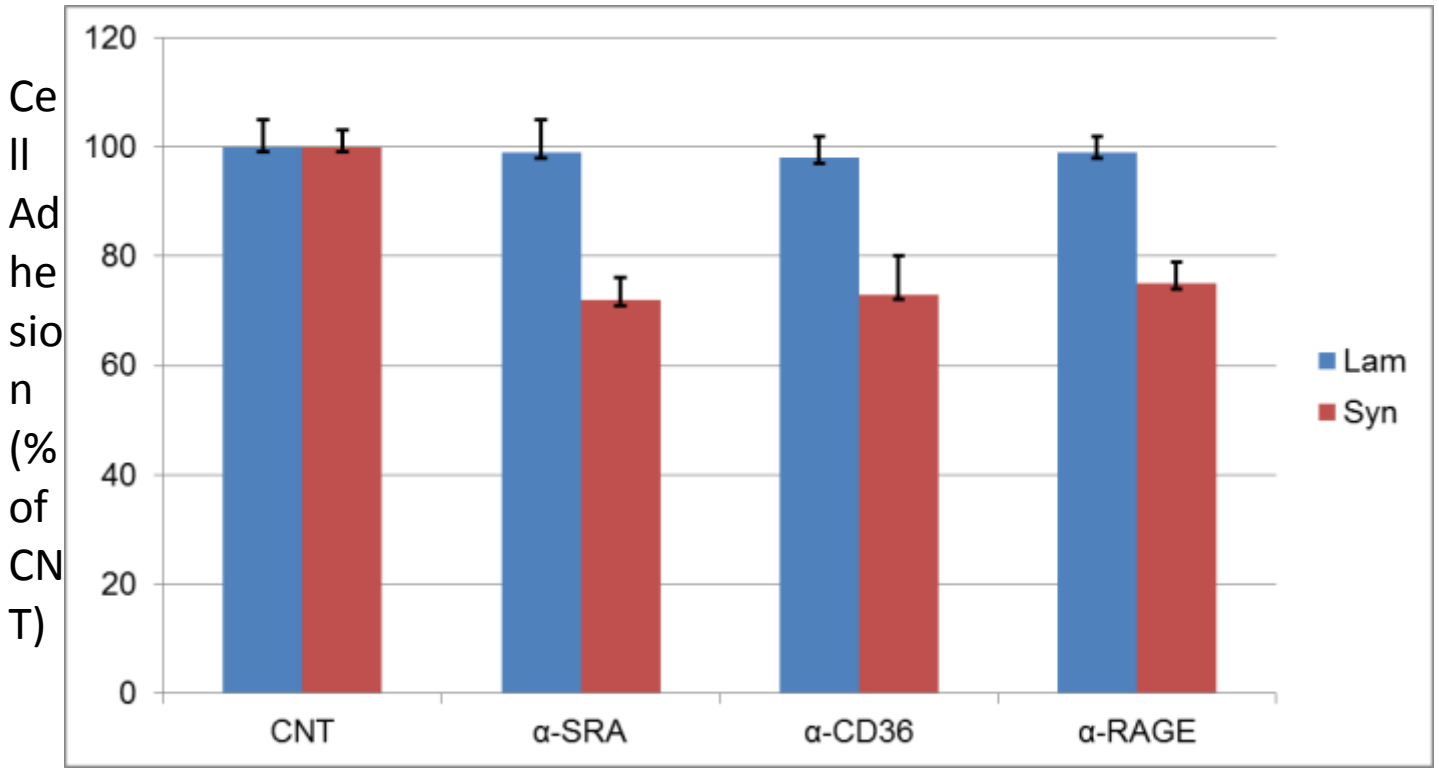
Figure 2c. Effects of anti CD11b on cell adhesion to lam and lam/α-syn-coated surfaces.



50,000 BV2 or N9 cells were added to each well of a 96 well plate as described in Fig. 2b. The indicated antibodies were then added to the appropriate wells and cell adhesion was assessed as describe in Figure 2b.

•**Effects of anti scavenger receptor antibodies on cell adhesion.** The fact that we only observed between 30-40% inhibition of cell adhesion to lam- or lam/α-syn–coated surfaces in the presence of anti- CD11b (Figure 2c) or anti-beta$_2$ (data not shown) antibodies suggested that other cell surface receptors may be playing a role in microglial cell adhesion to lam- or lam/α-syn–coated surfaces. We choose to examine scavenger receptors for a variety of reasons. There are many situations where the release and accumulation in the extracellular matrix of normally intracellular proteins can often lead to extracellular oxidation, oligomerization, and nitration of these proteins. This in turn could trigger neurodegenerative responses. Beta amyloid, oxLDL, and prions are examples of proteins that cause neurodegeneration when they accumulate and are modified in the extracellular compartment of the CNS. Previous work in our laboratory and others (see Prabhudas et al., 2014 for a review) have shown that many of the released extracellular proteins serve as ligands for scavenger receptors. We therefore examined whether antibodies directed against these scavenger receptors interfere with N9 cell adhesion to lam or lam/α-syn -coated surfaces. As shown in Fig 3a. antibodies directed against scavenger receptors had a significant effect (approximately 25%) on N9 cell adhesion to these protein-coated surfaces.

Figure 3a. The effects of antibodies against several scavenger receptors on N9 cell adhesion to lam- and lam/α-syn – coated matrices. See Figure 2 for details.



To ensure that these antibodies (anti-CD11b and anti-SR-B2) were not exerting a non-specific inhibition in our adhesion assay, we examined their effects on the adhesion of human skin fibroblasts to matrix bound lam and lam/α-syn surfaces. First of all, we did not observe any increased adhesion of human skin fibroblasts to matrix bound lam/α-syn surfaces over that seen on matrix-bound lam surfaces. Second, we did not observe any inhibitory effects of anti CD11b or anti-SR-B2 to these matrices (Figure 3b).

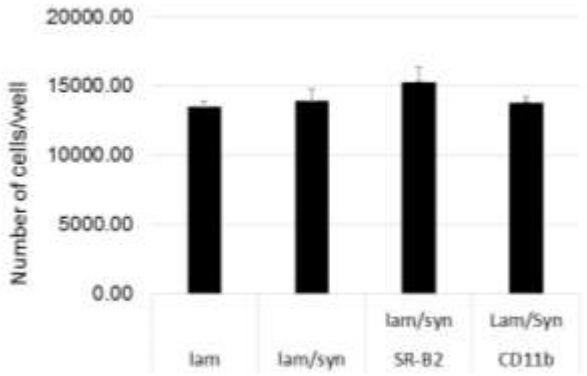


Figure. 3b. Effects of antibodies against SR-B2 and CD11b on human fibroblast cell adherence to lam or lam/α-syn-coated matrices. See Fig 2 for details.

To confirm the role of scavenger receptors in mediating cell adhesion to lam/α-syn coated surfaces we transfected CHO cells with adenovirus with the genes to encode for SR-B2 and assessed their capacity to adhere to lam or lam/α-syn-coated matrices. As shown in Fig. 3c, the number of adherent

CHO cells expressing SR-B2 to lam/α-syn -coated surfaces was three fold more adherent as compared to wild type vector transfected CHO cells. Control experiments revealed the there was no difference in SR-B2 expressing CHO adherence to matrix bound lam surfaces as compared to non-transfected cells (data not shown).

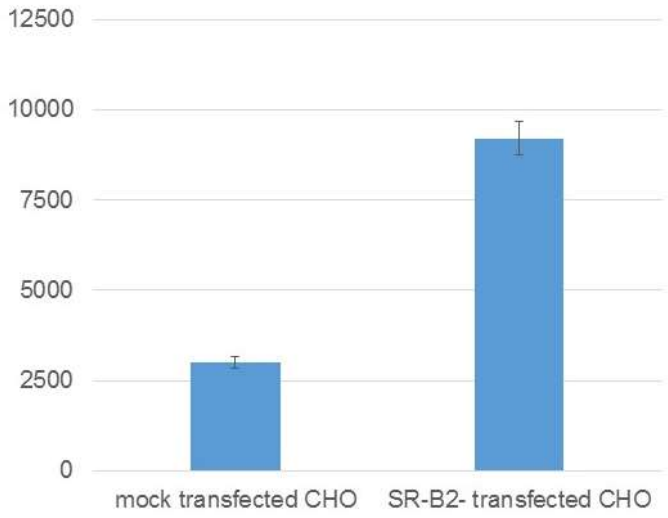
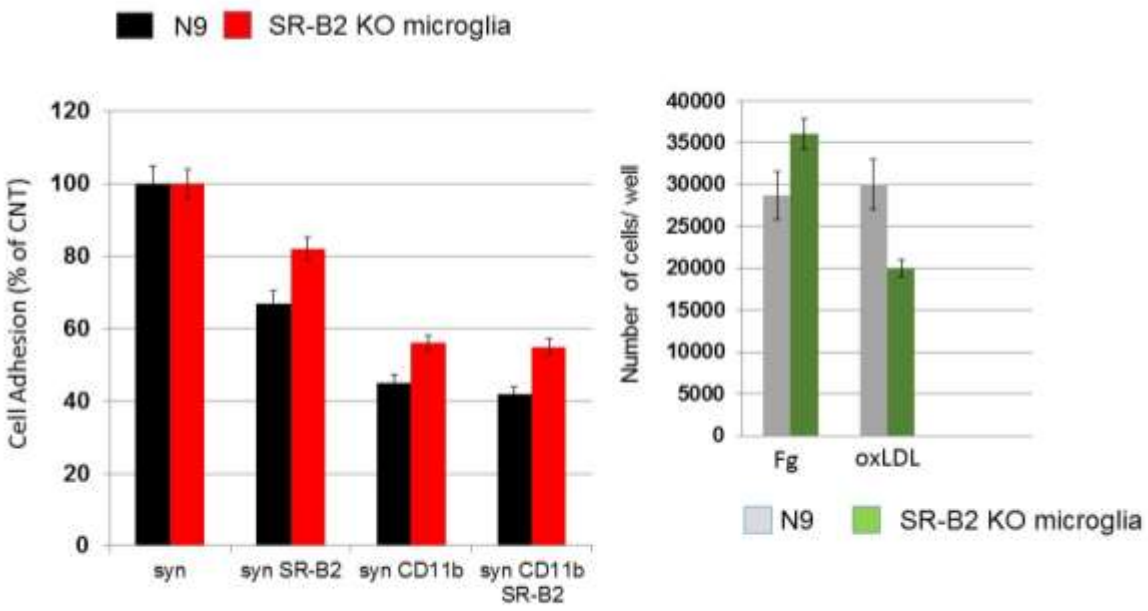


Figure 3c. Cell adhesion of CHO and SR-B2 transfected CHO cells onto lam or lam/α-syn-coated matrices. CHO and SR-B2 transfected CHO cells were obtained as prepared as described. See Fig 2 for details.

•Adhesion of immortalized adult primary microglia cells obtained from SR-B2 knockout mice.

We then obtained adult microglial cells from SR-B2-knockout mice and immortalized them as described (manuscript in preparation). As shown in Figure. 4, these SR-B2 cells exhibited a reduced level of adhesion to matrix-bound lam and lam/α-syn surfaces. In addition, the anti-SR-B2 antibody was less inhibitory on the adhesion of SR-B2-KO microglia cells as compared to the effects of this antibody on N9 microglial adhesion to matrix bound lam/α-syn (Figure 4). The failure to fully restore cell adhesion of SR-B2 KO microglia cells to lam/α-syn is probably due to the presence of other scavenger receptors (SR-A and SR-J or RAGE) as well as the presence of CD11b expressed by these cells. To further confirm the lack of SR-B2 on SR-B2 KO microglia cells, we stained these cells with fluorescent labelled antibodies directed against SR-B2 and observed no fluorescence above background (data not shown). Additional control experiments show that there is virtually no difference in the adhesion of N9 and SR-B2 KO microglia cells to fibrinogen (a ligand for CD11b) (Loike et al., 1991) . In contrast, SR-B2 KO microglia cells do not adhere as well to oxidated LDL-coated surfaces (a ligand for SR-B2- Prabhudas et al., 2014) as compared to N9 cells.

Figure 4.


N9
SR-B2 KO microglia
Cell Adhesion (% of CNT)
120
100
80
60
40
20
0
syn
syn SR-B2
syn CD11b
syn CD11b SR-B2
Number of cells/ well
40000
35000
30000
25000
20000
15000
10000
5000
0
Fg
oxLDL
N9
SR-B2 KO microglia

In summary, our adhesion data support the hypothesis that CD11b integrins and SR-B2 (CD36) mediate adhesion of N9 and BV2 microglia cells to matrix bound α-syn surfaces.

•N9 chemotaxis

We observed that native N9 cells are poorly chemotactic in response to a variety of chemoattractants, such as GMCSF or MCP-1 (data not shown). Incubating these cells with GMCSF triggered only about <1% of the cells to migrate across a naked filter in cell insert chemotactic chambers. However, those cells that did migrate only migrated to the other side of the filter but did not detach from the filter to the bottom of the lower compartment. To detach these cells from the filter, we briefly treated them with 5 mM EDTA for 5 mins which detached all the cells from the underside of the filter into the bottom chamber. We did not observe by microscopy any cells from the upper side of the filter passing across the filter in response to this treatment (data not shown). Those few N9 cells that migrated were maintained in culture for several days to allow for proliferation. Once sufficient numbers of cells proliferated we recovered those cells and examined their chemotaxic properties. We observed that, in fact, they exhibited enhanced migratory behavior in response to GMCSF.

We next examined the migratory response of this selected population of N9 cells across naked filters, filters coated with lam and filters coated with lam/α-syn. N9 cells that migrated once through the chamber showed about a 10 fold increased chemotaxic activity in response to GMCSF through lam coated filters as compared to non-selected N9 cells (Data not shown). The presence of GMCSF in the bottom compartment increased the number of migratory cells by almost three fold resulting in about 10% of the cells migrating with 24 hr. (Figure 5).

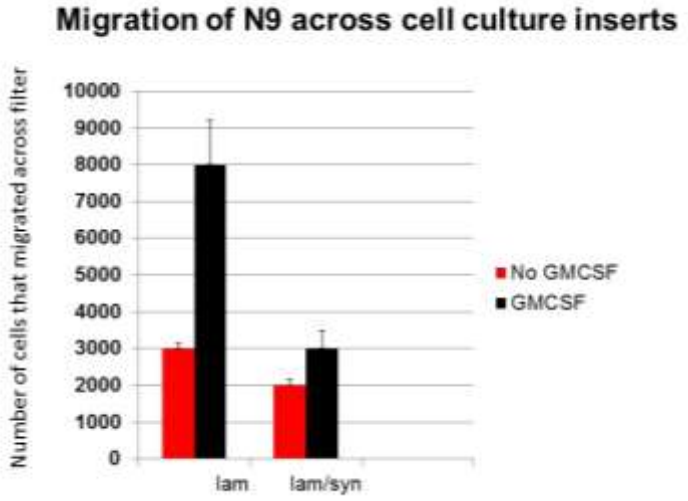


Figure 5

The presence of α-syn on the filters dramatically reduced the number of migrating N9 cells by 63% in response to GMCSF (Figure. 5). When we examined the effects of anti CD11b and SR-B2 on chemotaxis of N9 across laminin/α-syn coated filters, both antibodies further reduced migration (Figure. 6). Anti SR-B2 antibodies blocked chemotaxis by 35% whereas anti- CD11b inhibited chemotaxis by 55% (Figure. 6). The combination of adding both antibodies did not further reduce

inhibition. As expected anti-SR-B2 antibodies had a minimal effect on the blocking the migration of SR-B2 KO microglia cells across laminin/α-syn coated filters in response to GMCSF (Figure. 6). In contrast, anti-CD11b antibodies continued to block chemotaxis of SR-B2 microglia cells but not as robustly as their effects on N9 cells (Figure 6).

Figure 6. Chemotaxis across laminin/α-syn (% of CNT)

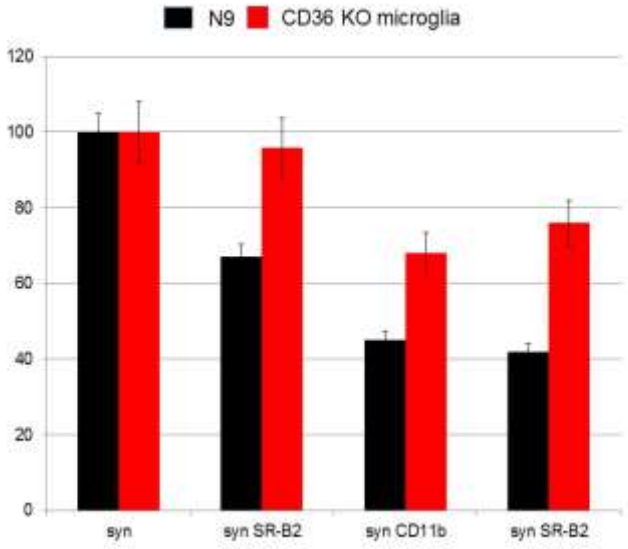


Figure 6

•Effects of Ursolic acid on N9 chemotaxis.

Ursolic acid has been reported to block several effects of SR-B2 (Ikeda et al., 2007; Wilkinson et al., 2011). We therefore examined its effects on chemotaxis across lam/α-syn -coated filters in response to GMCSF (Figure 7). Surprisingly, incubating cells with 1-10 uM of ursolic acid significantly reduced chemotaxis of N9 cells across lam/α-syn –coated filters while not affecting only chemotaxis of SR-B2 microglial cells (Figure 7). Thus, in contrast to anti-SR-B2 antibodies that prevent reverse the inhibition of chemotaxis across α-syn, UA further reduces chemotaxis across α-syn. However, control experiments reveal that UA also inhibits chemotaxis of N9 cells across lam – coated filters suggesting that it blocks SR-B2 mediated chemotaxis. In cells lacking SR-B2 there was no effect of UA or anti – SR-B2 antibodies on chemotaxis (Figure 7)

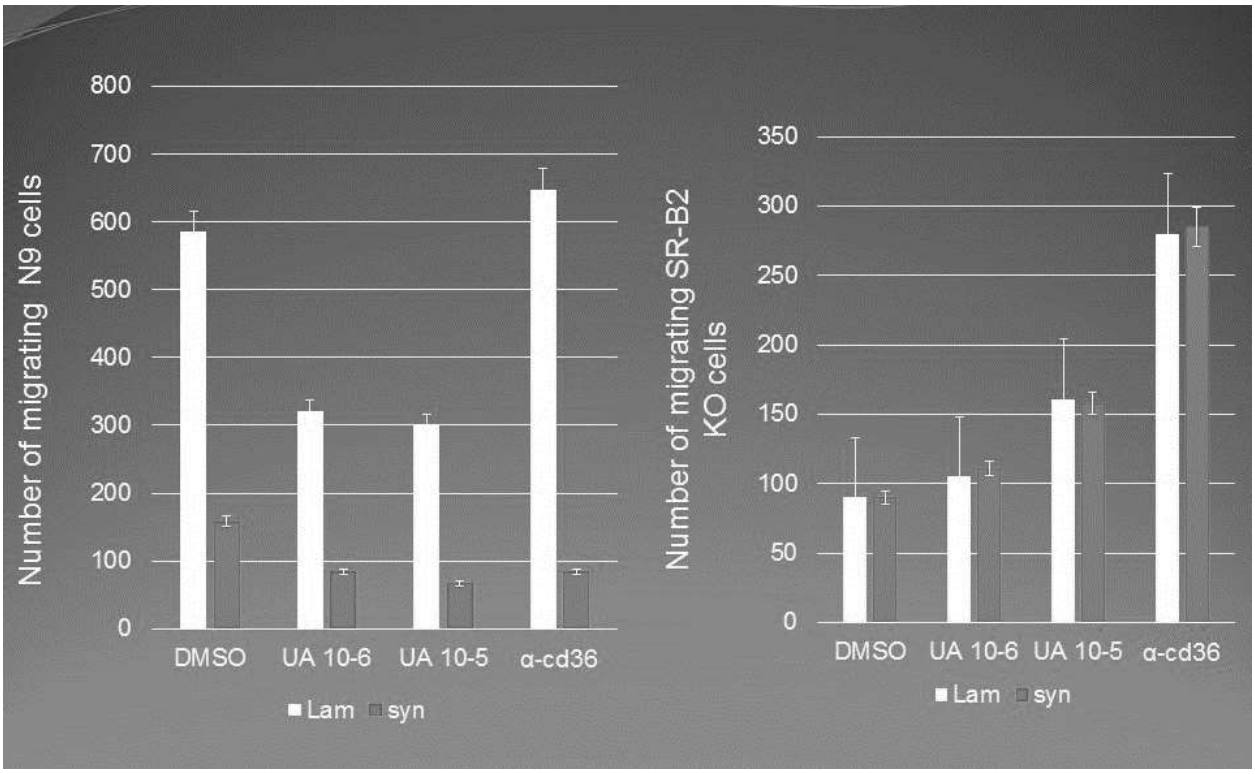


Fig. 7. Effects of Ursolic acid (UA) on N9 chemotaxis. N9 cell chemotaxis across filters coated with either lam or lam/ α-syn were assessed as described under Materials and methods. 100,000 N9 cells were added to the upper compartment of cell culture inserts and GMCSF was added to the lower compartment. Chemotaxis across the filters was measured after a 24 hr incubation was measured as described under Materials and methods.

•**Clearance of fluorescent-labeled α-syn by N9 and SR-B2 KO microglial cells**. Microglial cells are known to serve as sanitation engineers, clearing extracellularlly released proteins such a beta amyloid (Kopec and Carroll 1998). The failure to clear extracellularly released beta amyloid is thought to contribute to the development of Alzheimer's disease (Kopec and Carroll 1998). We therefore speculated that microglial cells might also clear and process α-syn. We first labeled α-syn with Alexis 488 as previously described (REF). We then coated lam-coated 96 wells for 24 hr with buffer containing 1 µg of labeled α-syn with 4 ug of unlabeled α-syn. Cells were then added to each well for 2 hr and then each well was washed with PBS and all adherent cells were removed by the addition of 5 mM EDTA. The remaining fluorescence was then assessed by a cytofluor cell plate reader as described under Materials and Methods. Control experiments by microscopy showed that EDTA effectively removed all the cells from the wells and that the addition of EDTA did not by itself release labeled α-syn from the wells. After a two hour incubation with N9 cells, about 30% of the fluorescence was removed from the wells. In contrast, only 20% ($p<0.05$) of the fluorescence was removed from the wells incubated with SR-B2 KO microglial cells (Figure. 8).

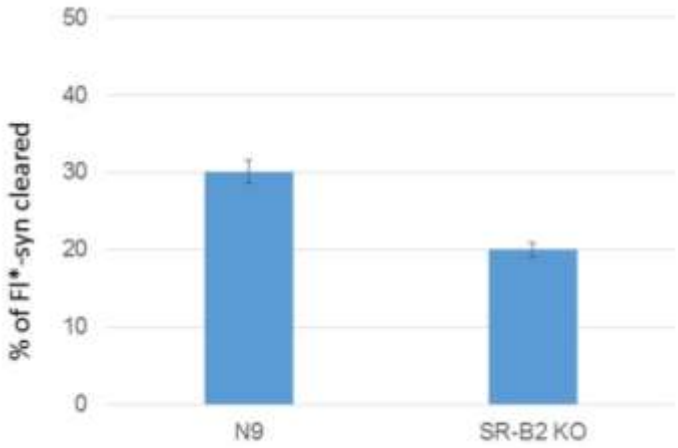


Figure 8. Clearance of matrix-bound α-syn by N9 and SR-B2 KO microglia cells. Fluorescently labeled α-syn was prepared as described under Materials and Methods and 96 well plates were precoated with lam and then coated with 1µg/well of fl- α-syn and 4 µg/well of unlabeled α-syn. 50,000 N9 or SR-B2 KO microglia cells were then added to the wells for 2 hrs. At that time the cells were detached with 10 mM EDTA and the remaining fluorescents measured in each well.

•SR-B2 Immunostaining in Human SN.

SN tissue was obtain post mortem from PD patients (N=4) and their age-matched controls (N=4). Using an antigen retrieval protocol (see key accomplishments) we noted selective SR-B2 immunostaining in the human SN from PD patients and no staining in SN from age matched controls. Most SR-B2 immunostaining in these PD tissues seem to be localized to the SN microglia based on morphology (Figure 9).

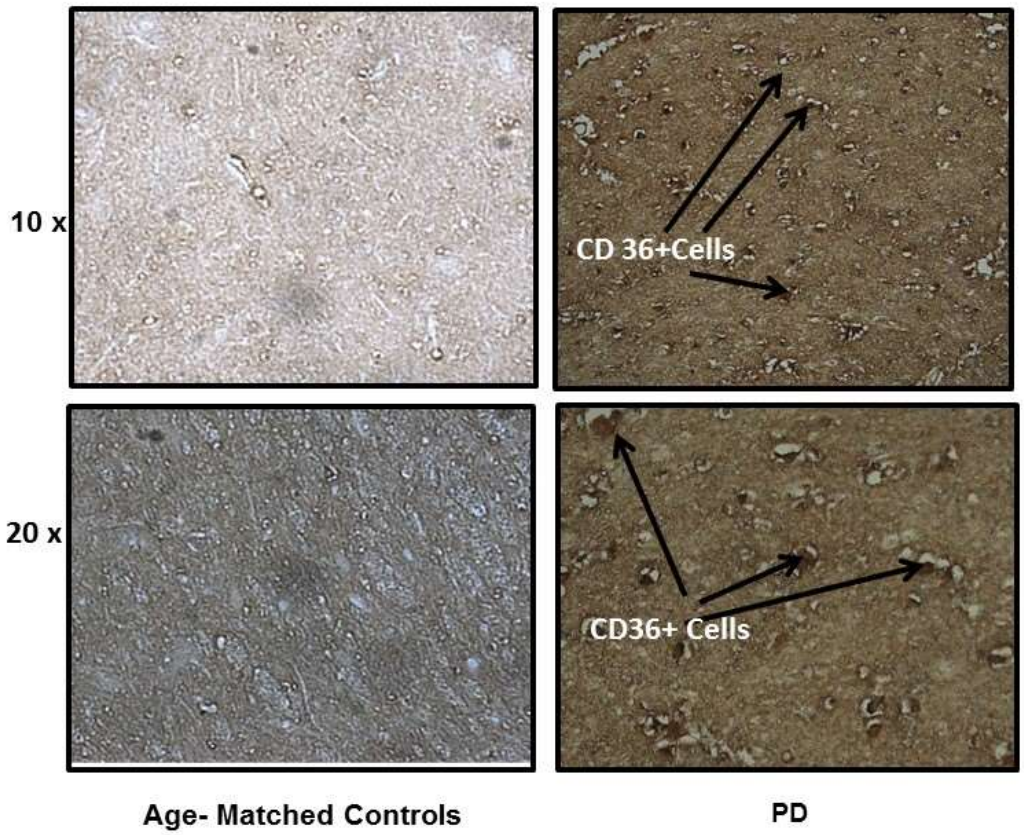


Figure 9. CD36+ Immunostaining in the Human Parkinson Disease Substantia Nigra versus Aged-Matched Controls. Eight micron thick paraffin sections of the substantia nigra pars compacta (SNpc) from PD and aged-matched controls were deparaffinated in xylenes and alcohols then immunostained for CD36 using a rabbit polyclonal antibody and visualized using Diaminobenzene. Pictographs show little or no CD36 + immunostaining on both 10x and 20x views of controls whereas the PD tissues exhibit strongly positive immunostaining for CD36 on both the 10x and the 20x views

Discussion

A hallmark pathological feature of PD is neuroinflammation which we believe probably underlies the progressive nature of the disease. Thus, our goal is to connect synuclein to the initiation of the inflammatory response in the SN and to the reactive microglia in this area of the brain. The noted inflammation has been shown to produce cytokines and chemokines which, most likely, alter the environment of the already fragile DA neurons in the PD setting. Cell signaling via pattern recognition receptors (ref, Khang et al 2005) on the surface of the microglia read the changes in the SN environment, which can occur when dying DA neurons release their contents into the extracellular space (Dauer and Przedborski, 2003). Although synuclein is a ubiquitous protein, in terms of PD, it may acquire a toxic gain-of-function nature, particularly if the gene exhibits duplication or triplication (Singleton et al, 2003; 2004). In this respect, its toxicity in the SN environment may also be at the root of synuclein aggregates. In our studies using immortalized N9 microglia, we noted that the addition of syn to wells containing microglia increased adhesion among microglia to each other. The microglia

tended to adhere to one another thus producing clumps or sheets of microglia. Along this line and, at the same time, the addition of syn decreased the migration of microglia while significantly increasing adhesion. It seems that adhesion and migration go together in many respects. Thus, the cells tend to be more stationary possibly by hindrance via syn, which could possibly facilitate syn aggregation as it seems that the lack of movement may contribute to an environment that may promote syn aggregation. Antibodies against the specific molecules involved here blocked the synuclein effect. Anti-beta-integrin blocked the adhesion to some degree (@30%) but not 100%, which indicates that other cell surface forces are involved. These might include CD18 and the toll-like receptors (Triantafilou et al, 2007).

Migration of microglia is crucial to several brain functions including development, after damage, and in disease states involving inflammation. Based on the roles of invadosomes in peripheral tissues, it might be that microglia use these invadesome structures to adhere to and to degrade the ECM which seems to be necessary for efficient migration (Vincent et al, 2012). Invadosomes are distinct from other adhesion complexes, and have been identified in normal cells, like leukocytes, osteoclasts and endothelial cells, that need to cross tissue barriers to fulfill their function. Invadosomes are subdivided into podosomes, found in normal cells, and into invadopodia specific for cancer cells (Brisson et al, 2012). Invadosomes function as pH regulators in physiological and pathological conditions, with a particular emphasis on ECM remodeling. These invadosomes have features similar to highly aggressive cancer cells. This means that they behave like tumor cells in that they can escape their primary residing area and invade the surrounding tissues in order to reach the systemic circulation. They are localized to the ventral membrane of cells that are grown under 2-dimensional conditions and are key structures in physiological processes such as inflammation and the immune response, bone remodeling, tissue repair, but also in pathological conditions such as osteopetrosis and the development of metastases. While these two structures exhibit differences in organization, size, number and half-life, they share similarities in molecular composition, participation in cell-matrix adhesion and promoting matrix degradation. A key determinant in invadosomal function is the recruitment and release of proteases, such as matrix metalloproteinases (MMPs), serine proteases and cysteine cathepsins, together with their activation in a tightly controlled and highly acidic microenvironment. Therefore numerous pH regulators such as V-ATPases and Na(+)/H(+) exchangers, are found in invadosomes and are directly involved in their functioning. However, to perform their functions during development and after central nervous system injury, the brain's immune cells (microglia) need to migrate through a dense neuropil and through the extracellular matrix (ECM) in order to degrade the ECM. How the ECM is degraded is not known at this time. Our migration data notes that syn significantly decreases microglial migration which would allow the syn on the surface of the microglia to interact with other syn-coated microglia thus possibly causing clustering of the microglia and syn aggregation. We noted that certain antibodies can partially block the syn-induced adhesion-migrationof microglia. Thus, other factors/receptors are involved. Part of the inflammatory response in the brain as a result of injury is the production of cytokines like TNF and reactive oxygen species (ROS). In the case of PD and in the MPTP mouse model of PD, the SN is ripe with activated microglia. In fact, CD11b or Iba-1 immunostaining following MPTP treatment and in the PD brain as well, one can see the area that the SN occupies while viewing only microglia (data not shown) as this was the only immunostaining performed here. In our synuclein transgenic mice, we noted that the microglial immunostaining in the SN showed a pattern of activated microglia that followed the pattern of the SN. The similarity of the syn response to the MPTP response indicates that syn in vivo can initiate an inflammatory response. This response includes the production of COX-2, iNOS, and IL1-β as well as reactive oxygen species (ROS) (Codolo et al, 2013). In adding syn to our microglial cultures and then performing an elisa panel of inflammatory markers, it was noted that these markers were found in our microglial-syn mix. Thus, syn can exert a

toxic effect through the stimulation of such markers and it is these markers that alter the cellular environment making it detrimental to the SN DA neuron.

One of the jobs of microglia is to clear released proteins from the extracellular space. Thus, microglia should clear any debris including syn that is relased from dying neurons in the SN. Hot off the presses data using synuclein-coated nanobeads show that these beads adhere to the surface of the microglia. If this is the case (and we do have to repeat these studies for clarification), since synculein coats the microglial cell surface, this would conceivably do one thing: promote the aggregation of synuclein via the cell surface communication between the microglia However, this remains to be demonstrated in our in vitro system. Our data demonstrate that syn may contribute to the development of PD through the behavior of microglia that have been activated. Furthermore, we have demonstrated the prominence of CD36 in tissues from PD patients. We are now preparing two manuscripts for publication and preparing a grant to continue these exciting studies.

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